THE EFFECT OF SALINITY ON *RHIZOBIUM* SURVIVAL, NODULE FUNCTION AND NODULE FORMATION IN THE SOYBEAN-*RHIZOBIUM JAPONICUM* SYMBIOSIS

A DISSERTATION SUBMITTED TO THE GRADUATE DIVISION OF THE
UNIVERSITY OF HAWAII IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

IN AGRONOMY AND SOIL SCIENCE

AUGUST 1982

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ACKNOWLEDGEMENTS

This work was supported in part by Grants AID/DSAN-G-0100 (211-d) and AID/ta-C-1207 (NifTAL Project) from the United States Agency for International Development. The conclusions reached within this study do not necessarily represent the views of the granting agency.

ABSTRACT

Symbiotic nitrogen fixation may be adversely affected by saline environments. This dissertation describes experiments that assess the salt sensitivity of: 1) Rhizobium as free living organisms; 2) soybean nodule function; and 3) soybean nodule formation. In addition, a split-root plant growth system is described which can be used to separate the effects of salinity stress on host yield potential from the effects of salinity on nodule processes.

The growth rate of Rhizobium in culture media is slowed by the addition of NaCl. Some strains were incapable of growth at the highest level of salt used (120 mM NaCl). However, all withstood substantial osmotic shock and most survived for extended periods in saline solutions equivalent to the concentration of sea water. The results show that the effects of increasing moisture tension and salinity on Rhizobium survival in soil are additive.

By independently subjecting nodules and shoots to salinity stress it was possible to show that the soybean-Rhizobium japonicum nodule system was not greatly affected by exposure to 120 mM NaCl. The main reduction in nitrogen fixation was the indirect effect of salinity on leaf expansion, shoot yield potential and the sink for nitrogen.

The early processes of nodule formation were extremely sensitive to NaCl in the rooting medium. When only 26.3 mM NaCl was added to the nutrient solution two hours prior to inoculation, nodule number and mass were reduced by 50% and 79.9 mM NaCl reduced nodule number,

mass and nitrogen fixation to less than 10% of the controls. Rhizobium japonicum, reisolated from nodules from the high salt treatment did not form more nodules under saline conditions than isolates from controls.

By independently subjecting the various processes of the symbiosis to salinity stress it was determined that the early steps in nodule formation are the most sensitive to salinity. This sensitivity indicates that high quality irrigation water must be used during the establishment of symbiotic legumes.

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CHAPTER I

INTRODUCTION

There are an estimated 4×10^6 km^2 of saline lands in the world today. This figure does not include large areas of the world's major deserts nor areas where agricultural practices have generated saline soil conditions (secondary salinization) (Flowers et al., 1977). Salinity limits production on 25% of irrigated land in the USA (Carter, 1975). Secondary salinization can be considered to be a more acute problem since this process results in either the loss or reduced productivity of improved land.

There are two approaches to solving salinity problems. The first involves management practices to reduce the level of salts in the root zone as outlined by the U. S. Salinity Laboratory (U. S. Salinity Laboratory Staff, 1954). The second approach requires manipulation of the biological component of agricultural systems to find plant material capable of economic yields under ever increasing levels of salinity. The first approach is a temporary solution to the problem since many large irrigation projects end up recycling salts, which over time, increase salt concentrations in irrigation waters, and subsequently, they accumulate in the root zone. The long term solution to crop production under increasing salinity lies in understanding and manipulating the mechanisms that enable some plants to grow under saline conditions. This requires an understanding of the physiology of the salt mediated growth suppression of glycophytes as well as an understanding of the mechanisms of salt tolerance found in halophilic

species. This knowledge combined with genetic manipulation of plants will enable desirable agronomic characters to be combined with salinity tolerance. Economic yields could thus be sustained in areas of naturally occurring salinity or secondary salinization.

Causes of Soil Salinity

The U. S. Salinity Laboratory (Staff, 1954) defines a saline soil as one in which the soil solution at saturation has an electrical conductivity (EC) of 4 mmhos/cm or more, and less than 15% exchangeable Na. Saline soils are generally not Ca deficient. The soluble salts can be of any species, however, Na salts and especially NaCl usually predominate.

Sources of soluble salts are varied. Most agricultural land becomes salinized as a result of fertilizer application and poor quality irrigation water. Arid conditions where evaporation rates are high and inadequate water is available for leaching result in concentration of salts in the root zone. Excessive accumulation of Na can compound the problem due to deterioration of soil structure and restricted drainage. High water tables in arid areas also can create saline soil conditions, through capillary rise of water and salts (Carter, 1975).

Growth Responses of Plants to Salinity

The tolerance of plant species to soluble salts varies. All marine plant life carries on photosynthesis and other metabolic functions at an electrical conductivity (EC) in excess of 40 mmhos/cm. (= 40 m eq dissolved salts/liter). Some terrestrial species grow in a salt

concentration near that of sea water as evidenced by the plant life at the sea water's edge. Most yields decline when crops are grown in the presence of much lower concentrations of salts. Despite large variation between crop species (Bernstein, 1964) none approach the tolerance shown by halophytes such as Atriplex vericaria which produced near normal yields in 700 mM NaCl (Black, 1960). Some halophilic alga can grow in 4 M NaCl (Johnson et al., 1968).

Shoot growth for many plants is inversely and linearly related to increases in osmotic pressure of the growth solutions: Sorghum bicolor (Patel et al., 1975); Glycine wightii (Gates et al., 1966; Wilson, 1970); Phaseolus vulgaris (Lunin and Gallatin, 1964); Glycine max (Able and Mackenzie, 1964; Bernstein and Ogata, 1966); Wheat, (Aceves et al., 1975) all show this type of response. The effect of salinity on plant growth is characterized by a decline in the ratio of shoot weight to root weight with increasing salt concentrations (Wilson, 1967; Wilson, 1970; Gates et al., 1966).

Glycophytes grown in saline conditions exhibit dramatic reduction in leaf area compared to controls (Wilson, 1970; Wignarajah et al., 1975). The reduction in leaf area of plants from salinity is due to both a reduced rate of cell division and cell enlargement. It is not, however, the internal accumulation of high levels of ions per se that limit leaf expansion. The main scheme of salt tolerance in holophytes is the accumulation of inorganic ions in the cell sap, while glycophytes tend to exclude excessive non-nutrient ions (Greenway, 1973).

Plant Water Relations and Salinity

According to early concepts of salt effects on plant growth, low soil osmotic potentials decrease water activity and water uptake by plants (physiological drought) which reduced cell turgor and cell expansion (U. S. Salinity Laboratory Staff, 1954). This has been shown to be incorrect since Bernstein (1961) and more recently Aceves et al., (1975) and Bower and Tamimi (1979) have shown that plants adjust their cellular osmotic potential to maintain a constant differential between the plant and soil solution. This differential is necessary if transpiration and turgor pressure are to be maintained. Salt stunted plants in moderately saline environments are not usually wilted since adequate turgor pressure is maintained (Kirkham et al., 1974).

Osmo regulation is the process whereby plants maintain potential gradients between their tissues and environment. This regulation is due to the accumulation of both inorganic and organic solutes and the subsequent transport of these solutes within the plant. Plants subjected to low matric potential cannot accumulate soil ions in sufficient quantities, so osmo regulation results from the synthesis of organic solutes more than plants under osmotic stress. Glycophytes under osmotic stress tend to maintain water potential gradients more with organic acids, especially malate, proline, and some ammonium compounds than do halophytes (Bar-Nun et al., 1977; Flowers and Hall, 1978; Wignarajah et al., 1975; Storey and Jones, 1977).

Storey and Jones (1977) examined 14 plant species with a wide range of salt sensitivities. They found that all species adjusted to

osmotic stress by accumulation of Na salts. Betaine was found in significant quantities in halophytes and semi-resistant species following salinization and proline was accumulated in all species. Flowers and Hall (1978) found that betaine accumulated rapidly when NaCl was added to the halophyte Suaeda maritima. These compounds can also serve as energy sources and precursors for the synthesis of other organic substances following the removal of the stress. Bar-Nun and Mayber (1977) found that proline content increased in Tamarix that was salt stressed and water stressed. Proline accumulation, however, was less and Cl^- accumulation greater when the water potential was lowered with NaCl as compared to polyethylene glycol, an osmoticum, that is not taken up by the plant.

Part of the osmo regulation process involves differential translocation of ions and solutes to various plant parts and to cell compartments. Wignarajah et al., (1975) found that Phaseolus vulgaris when transferred to 48 mM NaCl solution first accumulated high concentrations of Na in the first trifoliate along with higher concentrations of glucose and inositol than the leaves of control plants. All these solutes declined subsequently; Na was translocated out (to the stem), the sugar and sugar-alcohol probably was metabolized. The first trifoliate also tended to accumulate more K and Ca when salt was applied to the media. Salinized Glycine wightii (22 varieties) partitioned relatively more Cl than Na to the shoots. The ability to regulate ion content was associated with salt tolerance. More tolerant cultivars tended to exclude Na from the shoot to a greater degree than less

tolerant varieties. (Gates et al., 1970). Sodium is accumulated in the roots of many glycophytes in concentrations sufficient to account for much of the osmotic adjustment of this tissue (Bernstein, 1963). Glycophytes excluding Na from leaves tend to accumulate K, Cl and organic solutes in the shoots. These ions may also mediate other plant processes involving turgor such as stomatal movement.

Photosynthesis and Gas Exchange

Jensen (1975a, 1975b, 1976, 1977) has clearly shown that net photosynthesis per unit leaf area by Phaseolus vulgaris declines 22% with an osmotic potential of -3.9 atm compared to controls (-.4 atm). This decline was correlated with an increase in leaf resistance (r_l) and mesophyll resistance (r_m) (Jensen, 1975a). One-third of the reduction in net photosynthesis (Pnet) was due to r_l and two-thirds due to increasing r_m. Dark respiration per unit fresh and dry weight of leaves declined significantly when plants were grown under saline conditions. Increased energy for ion pumping for synthesis of organic osmoticums then probably explains the decline in dry matter accumulation of salt stressed plants. Neither increasing irradiance (Jensen, 1975b), changing leaf temperature (Jensen, 1976) nor changing root temperature (Jensen 1975b) countered the effects of salinity on photosynthesis or diffusive resistance. Gale et al., (1967) showed that net photosynthesis could be increased in salinized bean and cotton plants by increasing the CO_2 concentration of the atmosphere. Salinized plants that were CO_2 fertilized had carbon exchange rates (CER) approaching controls without CO_2 fertilization. The fact that r_l and r_m increase with salt stress

and that CO_2 fertilization can overcome some of this stress suggests that the light reactions of photosynthesis are not sensitive to salts.

It has been proposed that the enzymes of halophytes were more tolerant of salinity than glycophytes (Greenway, 1968). Weinberg (1975), however, isolated 6 enzymes associated with the photosynthetic process in Pisum sativum. The specific activities of these enzymes were the same in salt stressed plants as in controls. Flowers (1972) compared 4 enzymes from Suaeda maritima and Pisum sativum and found that no in vitro differences existed for the tolerance to salt of the enzymes from the two species. Flowers and Hall (1978) found that PEP carboxylase from Suaeda maritima was inhibited by salt concentrations in vitro that were lower than those measured in plant cells. This suggests that salts are compartmentalized in vacuoles and other non-inhibitory osmoregulators maintain cytoplasmic water potentials at sufficiently low levels to maintain turgor. Betaine and proline additions up to 1 M failed to inhibit the function of PEP carboxylase.

Conclusions

Although salinity retards growth of most crop plants some other species can flourish in highly saline environments. Growth suppression is characterized by a reduction in leaf area, and lower shoot/root ratios.

Salts reduce carbon assimilation of glycophytes due to an increase in resistance to gas exchange, mostly in the mesophyll. Biochemical processes and enzyme activities do not appear to be sensitive in the range of osmotic potentials which inhibit plant growth. Halophytes,

therefore, differ from glycophytes in that they can adjust osmotically to high concentrations of salts by ion uptake, then isolate or protect plant processes from these accumulated salts.

Since Pnet of legumes grown in saline environments is reduced it is not surprising that total symbiotic N accumulation is also reduced (Wilson, 1970). Symbiotic legumes, however, have additional processes and requirements that may be affected by salinity stress. For example, survival and growth of the Rhizobium, infection of root hairs and root-nodule development, and the functioning of the nodule enzyme system may be differentially susceptible to soluble salts. The purpose of the research presented in this dissertation was to identify salt susceptible steps in the soybean-Rhizobium japonicum symbiosis.

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CHAPTER II

EFFECT OF SALINITY ON GROWTH AND SURVIVAL OF *RHIZOBIUM*

Abstract

This study examines the effect of salinity on the growth and survival of *Rhizobium* in culture media and soil. Eleven isolates from saline and non-saline environments were compared. The growth (mean doubling time, MDT) of all strains and species tested decreased when the electrical conductivity (EC) of the culture medium (yeast mannitol, YEM, medium) was raised from 1.2 mmhos cm^{-1} to 6.7 mmhos cm^{-1} (15% sea water equivalent) and 13.1 mmhos cm^{-1} (28% sea water equivalent). Three out of 11 strains failed to grow at 13.1 mmhos cm^{-1}. Although growth was affected by salinity, four selected strains could survive in extremely high concentrations of salt. Two salt-sensitive and two salt-tolerant strains from the growth rate study were inoculated into solutions with EC's up to 43.0 mmhos cm^{-1} (92% sea water equivalent). Not only did all four strains survive the initial osmotic shock (5 hours after inoculation), it was not until 27 days after inoculation that the sensitive strains exhibited a significant reduction in viable numbers. The salt-tolerant strains survived for over 65 days with no reduction in viable counts. The interaction between soil moisture tension and soil salinity on *Rhizobium* survival in gamma irradiated soil was also examined. Six treatment combinations ranged from -0.1 bars and 0.2 mmhos cm^{-1} to -15 bars and 12 mmhos cm^{-1}. Sensitive strains declined from 10^7/g to 10^5/g of soil after 84 days of incubation at -15 bars and 12 mmhos cm^{-1}. Tolerant strains

survived the same period with no loss in viable numbers.

Results of these experiments indicate that many strains of Rhizobium can grow and survive at salt concentrations which are inhibitory to most agricultural legumes. Research emphasis concerning the effects of salinity on symbiotic nitrogen fixation should, therefore, be directed to other aspects of the symbiosis than the survival of the Rhizobium.

Introduction

Excessive soluble salts affect more than 4×10^6 km^2 of the world's potentially arable lands (Flowers et al., 1977). The agricultural potential of these lands is generally not limited by lack of solar radiation or temperature and if managed properly, these lands can become productive. Measurements of soil salinity are commonly made by determining the electrical conductivity (EC) in mmhos cm^{-1} or equivalent osmotic pressure in bars of the soil solution at saturation (U. S. Salinity Laboratory Staff, 1954). Sea water at 20^o has an EC of 46.6 mmhos cm^{-1} (Thomas et al., 1934). Actual concentrations of soluble salts that are present in soil moisture films fluctuate with changes in soil water content.

Little work has been done concerning the effect of salinity on the legume-Rhizobium symbiosis. That nitrogen accumulation by the symbiotic systems of soybean, alfalfa and Glycine javanica is reduced by salinity has been well documented (Bernstein and Ogata, 1966; Steinborne and Roughley, 1975).

Saline conditions, may limit the symbiosis by: 1) affecting survival and proliferation of Rhizobium in the soil and rhizosphere; 2) inhibiting the infection process; 3) directly affecting root-nodule function; and/or 4) reducing plant growth, photosynthesis and demand for nitrogen. Since soil salinity may directly affect either symbiont or affect their interaction it is essential to identify the processes most sensitive to salinity. Efforts then may be directed toward improving the tolerance of the most sensitive symbiont or process of the symbiosis.

Reported effects of salts and soil moisture tension in the literature are mixed. Enhancement of growth by Rhizobium spp. in media with 1% NaCl (approximately 19 mmhos cm^{-1}) was reported by Pillai and Sen (1973). Steinborne and Roughley (1975) showed a reduction in growth rates of R. trifolii and R. meliloti in the presence of salt. Bhardwaj (1972) claimed that isolates from non-saline soils could not proliferate or survive salt affected soils. Carr and Ballard (1979) found that a strain of R. trifolii was able to withstand short exposure to fertilizer solutions with EC's in excess of 60 mmhos cm^{-1}.

This paper compares rhizobia isolated from salt affected soils with random inoculum strains from non-saline areas for their ability to grow and survive in salt solutions and in saline soil with different moisture tensions. Salinity treatments were, for the most part, selected to encompass a range of tolerance found in agricultural legumes.

Although salinity slowed growth of all strains tested in these experiments, there was considerable variation within the species tested

for tolerance to salt. Salt tolerance of the strains was not related to their ecological origins nor their growth rates in normal media. Rhizobia were able to withstand large changes in osmotic concentrations with little reduction in viable numbers. Sensitive strains were only affected after 5 days exposure to salt solutions which approached the concentration of sea water. There appears to be no interaction between soil moisture tension and salinity in relation to rhizobial survival in clay soil. A reduction in total water potential, whether due to osmoticum or high moisture tension, is the factor that affects rhizobial survival. Both "tolerant" and "sensitive" strains are much more tolerant of soil salinity and dry soil conditions than are their leguminous hosts.

Material and Methods

Source of Cultures

Isolates of rhizobia were made from legumes growing on beach sands and salt affected irrigated fields on the island of Oahu. Many of the isolates from beach sands were made close to standing sea water. Rhizobium isolation and plant infection tests were carried out as described by Vincent (1970). All isolates were confirmed as rhizobia on Macroptilum lathyroids except 17E which was confirmed on its host, Leucaena leucocephala. Sources of standard cultures from non-saline soils are given in Table II-1. All cultures were maintained on yeast extract mannitol, YEM, slants (Vincent, 1970).

Enumeration

Viable counts were performed using standard serial dilutions and plated by the drop plate count method (Vincent, 1970). The entire content of incubated soil vials were diluted; solution cultures were sub-sampled. All serial dilutions of salt treatments were made using isotonic diluents.

Effect of Salt on Growth

Inocula of 11 strains were diluted and one ml of each diluted culture was added to tubes containing 9 ml YEM broth ammended with either 0 (1.2 mmhos cm^{-1}), 50 mM NaCl (6.7 mmhos cm^{-1}) or 100 mM NaCl (13.1 mmhos cm^{-1}). Initial cell density was 1 x 10^3 viable cells ml^{-1}. Mean doubling times (MDT) were determined by viable counts made 3 times during the log phase of growth of each strain. The relative tolerance of different strains were expressed as the ratio of MDT at 13.1 mmhos cm^{-1} to MDT at normal YEM broth (1.2 mmhos cm^{-1}).

Effect of Concentrated Saline Solutions on Survival of Rhizobia

Four strains from the growth rate experiment were selected: 17E (fast-growing, salt tolerant); Hawaii 5-0 (fast-growing, salt sensitive); 21A (slow-growing, salt tolerant); USDA 110 (slow-growing, salt sensitive). Cultures were grown in YEM broth, diluted to 1 x 10^7 cells ml^{-1} in solutions containing only YEM broth salts, then inoculated into tubes containing broth salts only (1.4 mmhos cm^{-1}), broth salts plus 233 me l^{-1} total salts NaCl + $CaCl_2$ (18.4 mmhos cm^{-1}), and broth

salts plus 564 me l^{-1} total salts NaCl + $CaCl_2$ (43.0 mmhos cm^{-1}). Proportions of NaCl and $CaCl_2$ were adjusted to maintain a constant Na activity (SAR) as defined by the U. S. Salinity Laboratory Staff (Thomas et al., 1934). Viable counts were made 5 hours, 5 days, 27 days and 65 days after inoculation.

Growth and Survival of Rhizobia in Saline Soil at Two Moisture Tensions

Wahiawa subsoil (Tropeptic Eutrustox, clayey kaolinitic isothermic) was air dried and passed through a 2 mm sieve. A moisture release curve was developed using hanging water columns and a membrane pressure plate apparatus (Childs and Collis-George, 1950). The soil was also calibrated for the EC when increasing amounts of $CaCl_2$ and NaCl were added at a constant Na activity (SAR=10). Air-dried soil samples each weighing 34.4 g (30 g dry soil at 65° C) were added to 120 ml glass vials with air tight polyethylene caps. Vials and soil were gamma irradiated with 6×10^6 rads, and inoculated with appropriate mixtures of salt solutions and dilutions of YEM suspension cultures of strains 17E, 21A, Hawaii 5-0 and USDA 110 to impose treatments of -.1 and -15 bars moisture tension and EC's of 0.2, 5.0 and 12.0 mmhos cm^{-1} in all combinations. Initial inoculum densities ranged between 1.5 and 5.2×10^6 viable cells $gram^{-1}$ of oven dry soil.

Viable counts were made at 3, 7, 24 and 82 days after inoculation for fast-growers Hawaii 5-0 and 17E and at 6, 29, 49 and 86 days for slow-growing strains USDA 110 and 21A.

Results

The addition of NaCl to YEM broth increased the mean doubling time (MDT) for all strains tested (Table II-2). Sensitive strains such as Hawaii 5-0, Web 48 and 8 failed to grow in the highest level of salt. Isolates from saline soils were not consistently more tolerant to salt stress than other isolates. Strain 8 did not grow at 13.1 mmhos cm^{-1} and 7B grew very slowly. TAL 425, an isolate from an acid soil, grew well at 13.1 mmhos cm^{-1}.

Figure II-1 shows that all four strains selected from the growth rate experiment (17E, 21A, Hawaii 5-0, USDA 110) were able to survive initial exposure (5 hours) to solutions with an EC of 43 mmhos cm^{-1}. Only after 5 days exposure of USDA 110 to solutions at 43 mmhos cm^{-1} and after 27 days exposure for Hawaii 5-0 was any significant decline in viability detected. Viability of tolerant strains, 17E and 21A, was maintained in all the treatment solutions for the duration of the experiment. Viable numbers in YEM broth cultures (no salt) declined over time.

Survival of strains USDA 110 and 21A was affected in soil but only at the most extreme treatment combination of 12 mmhos cm^{-1} and -15 bars moisture tension (Figure II-2). All strains grew slightly when exposed to less extreme conditions.

Discussion

The salt tolerance of each symbiont of the legume-*Rhizobium* symbiosis may differ. It is practical, therefore, to examine the tolerance of one symbiont in relation to the tolerance of the other. Table II-3 shows that despite a large range in tolerance to salts between species of legumes there are no agricultural legumes that can be considered as highly salt tolerant. Comparing the sensitivity of the microsymbiont, *Rhizobium*, with that of the legume host will then indicate the relative importance of efforts to increase rhizobial tolerance to salts in strain selection programs.

The growth of all rhizobia tested was slowed by the presence of NaCl (Table II-2). These results are contrary to those obtained by Pillai and Sen (1973) who showed that the growth rate of *Rhizobium* spp. increased with 1% NaCl added to broth media (EC ≅ 18.0 mmhos cm^{-1}). Steinborne and Roughley (1975), on the other hand, have shown that the growth of both *R. trifolii* and *R. meliloti* was slowed with the addition of salt.

Relative tolerances to salt are given in Table II-2 to allow comparison of the effect of salt on growth of strains with large differences in inherent MDT's. Large increases in MDT, or growth suppression was not observed for any of the strains until the broth EC reached 13.1 mmhos cm^{-1} (28% sea water equivalent). This solution is in excess of solution concentrations in which agricultural legumes can sustain an economic yield.

Slow-growing strains were not more tolerant to salt than fast-growing strains. Isolates from saline environments were not consistently more tolerant to salt than isolates from non-saline soils. Isolates 7B, 8 and 14E from saline environments, either did not grow, or grew poorly in broth with an EC of 13.1 mmhos cm^{-1}. Strain TAL 425, an isolate from an acid tropical soil, had a MDT at 13.1 mmhos cm^{-1} only 2.4 times that in normal media.

Subjecting two salt "tolerant" isolates (17E and 21A) and two "sensitive" strains (Hawaii 5-0 and USDA 110) to an extreme reduction in osmotic potential had no effect on survival over 5 hours (Figure II-1). The solution with an EC of 43.0 mmhos cm^{-1} has a salt concentration equivalent to 92% of sea water (Thomas et al., 1934). Since rhizobia can withstand large reduction in osmotic potential, they must be able to rapidly regulate and adjust their internal solute concentration. Vincent et al., (1962) showed that R. trifolii populations fell from log 4.35 to log $\leq$ 1.0 in 160 mM NaCl (EC $\simeq$ 16.0 mmhos cm^{-1}) solution following a 48-hour exposure. Suspensions were, however, equilibrated with an atmosphere of 20% relative humidity which reduced the water content of the suspension to minimal amounts and left cells exposed to pure NaCl. Our results agree with Carr and Ballard (1979) who found that a strain of R. trifolii could survive short term exposure to fertilizer solutions with EC's approaching 60 mmhos cm^{-1}.

Normal YEM broth cultures with initial densities in excess of 1×10^9 cells ml^{-1} lost viability over time; declining until numbers were equal to, or less than, numbers in most salt treatments.

two symbionts. While the host legume produces seed and enters dormancy at the onset of the dry season; its microsymbiont, the Rhizobium, in order to survive, must be able to encounter much higher levels of salts in the soil solution as the soil dries.

Examination of rhizobial tolerance to stress in liquid culture, therefore, should not begin with cell densities of 10^9 ml^{-1} since these numbers cannot be maintained even in ideal conditions.

There is an inverse relationship between soil moisture tension and salinity in micro-environments. Reducing soil moisture content necessarily concentrates salts in the soil solution. Inoculum placed into soil at planting encounters immediate fluctuations in soil water potential (the sum of matric and osmotic potentials). Treatment combinations selected to test the effects of both soil moisture tension and salinity on the survival of Rhizobium range from soil conditions which may be considered optimum for plant growth (EC = 0.2 mmhos cm^{-1} and -0.1 bar) to an extreme condition which is unacceptable for supporting adequate growth of agricultural legumes (EC = 12 mmhos cm^{-1} and -15 bars). None of the four strains tested (17E, Hawaii 5-0, USDA 110, and 21A) lost viability rapidly even with the most extreme treatment (Figure II-2). R. leguminosarum strain Hawaii 5-0 which was sensitive to salt in both the growth rate study and exposure to salts in solution grew slightly and survived well in this clay soil. Isolate 21A which showed good tolerance to solutions with EC's of 43.0 mmhos cm^{-1} lost viability over time with the combined effects of high salinity and dessication. The decline was not strictly due to the low moisture content since 21A survived well at -15 bars and 5.0 mmhos cm^{-1}. R. japonicum strain USDA 110 lost viability as a function of increasing stress from both the osmotic and matric components of soil water potential. The fast-growing isolate 17E was completely resistant to all levels of stress in this experiment.

Marshall (1964) and Bushby and Marshall (1977) concluded that slow-growing rhizobia tolerated dessicated sandy soil better than fast-growing species. All species survived dessication better when sandy soil was ammended with montmorilloinite. Our study shows that a strain's inherent growth rate does not determine its resistance to low soil moisture content or salinity. Both 17E and B73 survived the combination of high salinity and increased moisture tension in this clay soil better than slow-growing strains USDA 110 and 21A.

Mahler and Wollum (1980, 1981) showed that even clay soil at -15.0 bars moisture tension was detrimental to the survival of many strains of R. japonicum and R. leguminosarum. Their soil was, however, autoclaved for 270 minutes after which soil samples used for incubation of rhizobia were contaminated with other bacteria. Growth of other bacteria in the incubation vessels decreased as a function of decreasing soil moisture content (Mahler and Wollum, 1980). We have found that by maintaining sterile conditions in gamma irradiated soil none of the strains tested was affected by either soil moisture tension or salinity levels used in this study. There appears to be no differential effect of moisture tension and salinity on rhizobial survival. The reduced survival of USDA 110 and 21A seems to be caused by the additive effects of increasing salinity and moisture tension.

The results of the above experiments emphasize that many strains of Rhizobium not only can withstand, but may even grow at, salt concentrations in excess of those tolerated by most agriculturally important legumes. This is consistent with the life-cycle characteristics of the

TABLE II-1. -- Source of cultures.

Culture	Host	Soil Environment	Location	Source
17E[a]	*Leucaena leucocephala*	beach sand	Kualoa, Oahu	b
Hawaii 5-0	*Lens esulenta*	acid tropical soil	Molokai	c
7B[a]	*Indigofera suffruticosa*	beach sand	Kualoa, Oahu	b
Web 48	*Glycine max*	soybean field	Midwest, USA	d
TAL 425	*Vigna radiata*	acid tropical soil	Thailand	e
23B[a]	*Macroptilum lathyroides*	flood plain	Kahuku, Oahu	b
8[a]	*Mimosa pudica*	beach sand	Kualoa, Oahu	b
14E[a]	*Croatalaria mucronata*	irrigated cane field	Ewa, Oahu	b
TAL 426	*Vigna unguiculata*	acid tropical soil	Thailand	e
21A[a]	*Canavalia* spp.	beach sand	Laie, Oahu	b
USDA 110	*Glycine max*	soybean field	Florida, USA	f

[a]Isolate from saline soil, EC of saturation extract $\geq$ 5.0 mmhos cm^{-1}.
[b]This study.
[c]S. N. May, M. S. Thesis, University of Hawaii, 1979.
[d]B. B. Bohlool, M. S. Thesis, University of Minnesota, 1979.
[e]Culture collection, NifTAL Project, Paia, Hawaii.
[f]Culture collection, USDA, Beltsville, Maryland.

TABLE II-2. -- Effect of NaCl on the mean doubling time (MDT) of of 11 strains of Rhizobium.

Strain	Mean Doubling Time (Hours) 0 mM NaCl (1.2 mmhos)	50 mM NaCl (6.7 mmhos)	100 mM NaCl (13.1 mmhos)	Relative Tolerance[c]
17E[a]	2.7	5.3	8.4	3.1
Hawaii 5-0	3.9	11.8	N.G.[b]	---
7B[a]	6.0	7.7	69.7	11.6
Web 48	8.7	11.9	N.G.	---
TAL 425	7.4	7.5	17.4	2.4
23B[a]	6.2	7.9	10.1	1.6
8[a]	5.9	7.1	N.G.	---
14E[a]	7.4	7.8	69.0	9.3
TAL 426	8.4	7.5	26.4	3.1
21A[a]	10.3	12.6	17.6	1.7
USDA 110	5.5	6.3	27.2	5.0

[a]Isolated from salt affected soils.
[b]No growth.
[c]The ratio of MDT at 13.1 mmhos cm^{-1}: MDT at 1.2 mmhos cm^{-1}.

TABLE II-3. -- The salinity tolerance of some agricultural plants.[a]

Crop	EC[b]
Barley	16
Birdsfoot Trefoil	10
Sesbania	9
Soybean	9
Alfalfa	8
Clover	4
Kidney Bean	3
Peas	2

[a]Bernstein (1).

[b]EC (mmhos cm^{-1}) associated with 50% decline in yield.

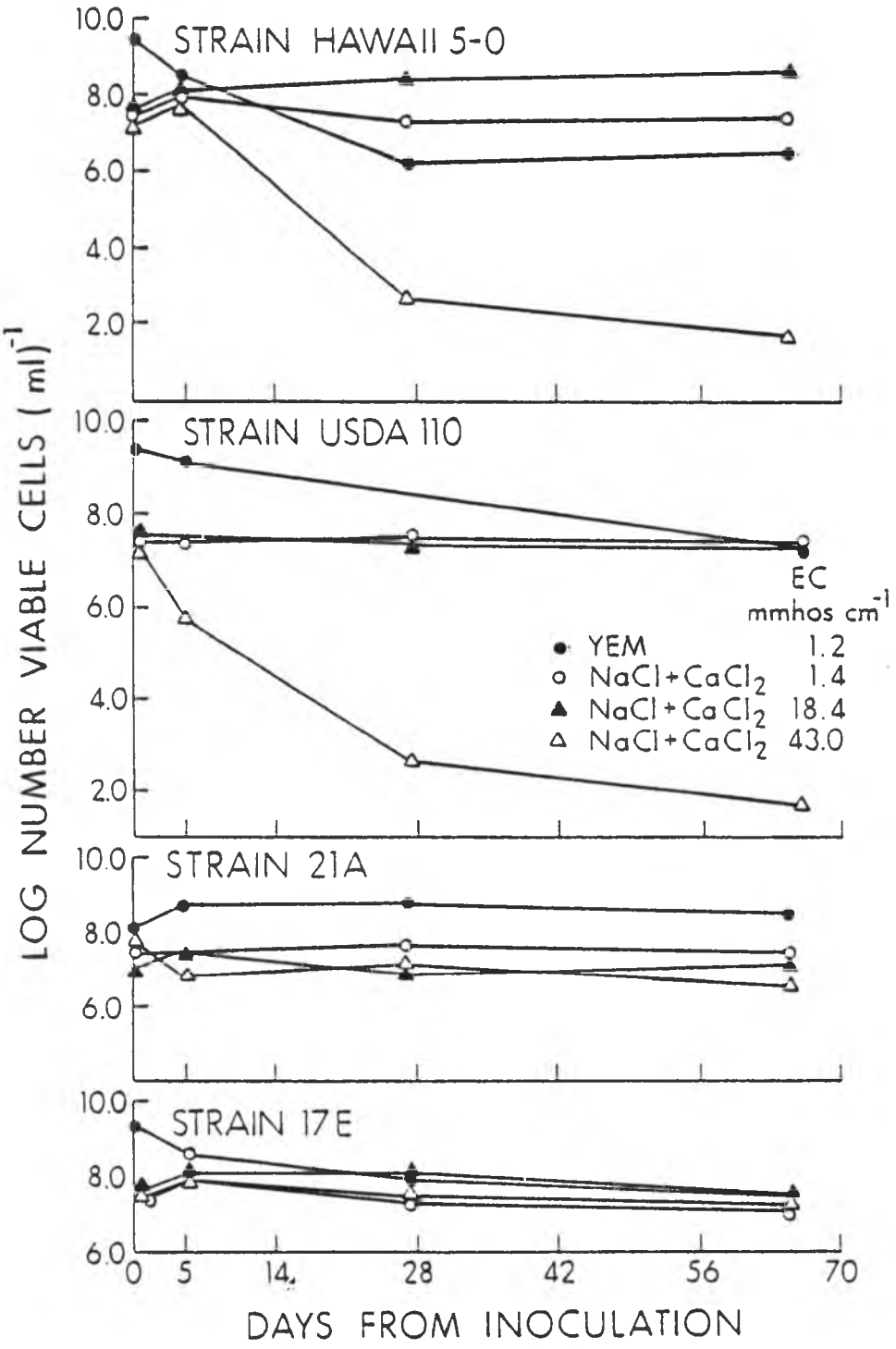

FIGURE II-1. -- Survival of Rhizobium in salt solutions.

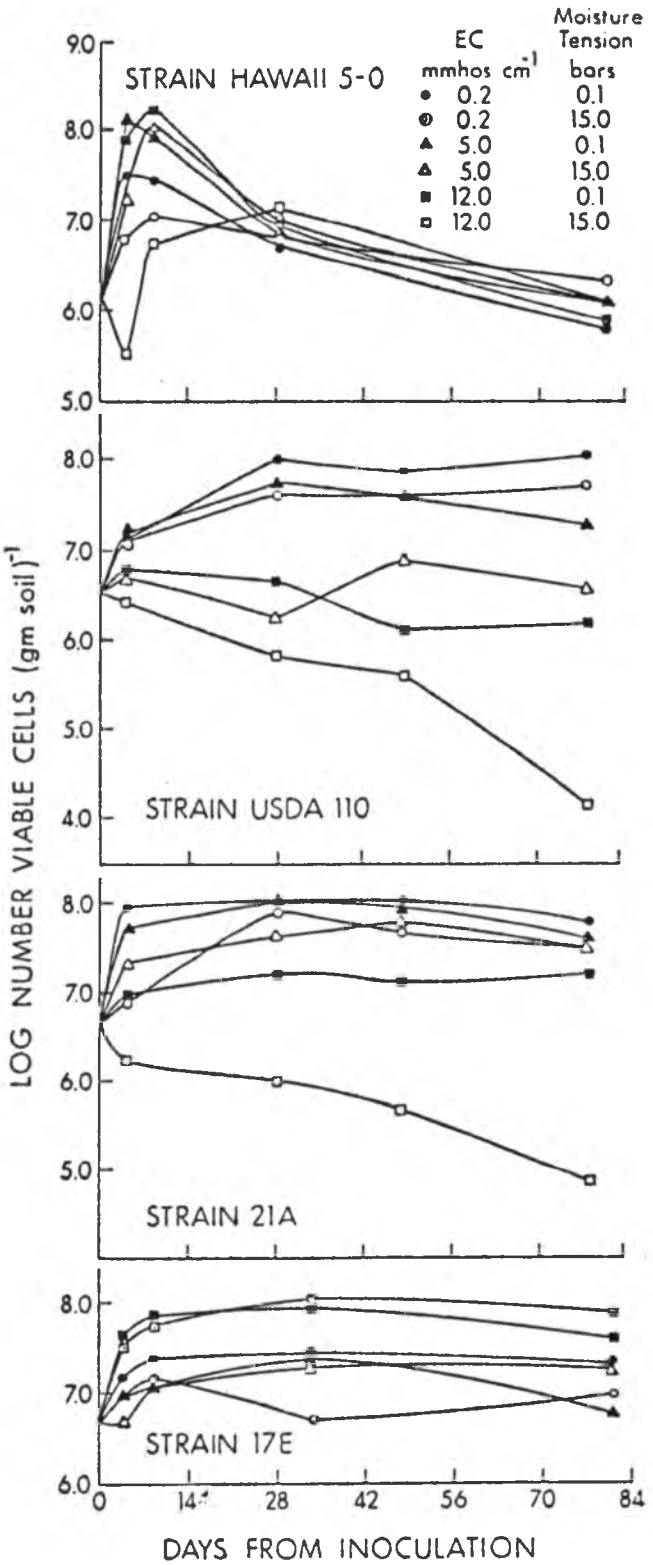

FIGURE II-2. -- Survival of Rhizobium in a salt affected Oxisol at two moisture tensions.

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CHAPTER III

A SPLIT-ROOT GROWTH SYSTEM FOR EVALUATING THE EFFECT OF SALINITY ON COMPONENTS OF THE SOYBEAN-*RHIZOBIUM JAPONICUM* SYMBIOSIS

Abstract

Study of the effects of salinity on symbiotic processes is complicated by the high degree of integration between shoot and nodules. Shoot photosynthetic capacity determines not only the amount of photosynthate available to the nodule, but the strength of the sink for nodule products. Salinity may also directly affect functional processes within the nodule.

Two experiments were undertaken which employed a split-root solution culture assembly that confined nitrogen fixation to one-half of soybean root systems. In one experiment, one-half of the root system was inoculated with non-nitrogen fixing strain (ineffective) of *Rhizobium japonicum*, SM-5, 48 hours prior to inoculating the other side with effective strain USDA 110. Nodulation and nitrogen fixation by USDA 110 when in combination with SM-5 was significantly suppressed compared to a second treatment where one-half of the root system was inoculated with USDA 110 and the other half remained uninoculated. Root development was significantly greater on the side producing effective nodules than that of the uninoculated or SM-5 nodulated half-root systems.

In another experiment, half-root systems were nodulated with USDA 110 and the opposite side with SM-5. After nodule development, NaCl (120 mM) was added to the nutrient solutions of: 1) neither half, 2) effective nodule (USDA 110) half, 3) ineffective nodule (SM-5) half,

or 4) both half-root systems. Osmotic potentials ($\Psi\pi$) of roots, nodules and leaves were measured. Leaves of plants with only half their roots salinized had $\Psi\pi$ (-10.3 bar and -11.1 bar treatments 2 and 3 respectively) comparable to the non-salinized control (-9.3 bar). Leaf $\Psi\pi$ of plants with both root halves in salt solutions was -17.9 bar. Nodules exposed to salt solutions had $\Psi\pi$ values ranging from -9.3 to -12.2 bar while $\Psi\pi$ of nodules in normal solutions ranged from -6.1 to -7.8 bar. Ineffective nodules always had a lower $\Psi\pi$ than similarly treated effective nodules. These results demonstrate that functional components of the soybean-Rhizobium japonicum symbiosis can be independently subjected to salinity stress. This methodology can be used to evaluate the relative sensitivity of the components of the symbiosis.

Introduction

The legume-Rhizobium symbiosis is a highly integrated system. Soil stress may act on the symbiosis indirectly by reducing plant growth potential and the availability of photosynthate, or act directly on nodule function and/or the infection process. In order to increase tolerance of the symbiotic system to a stress, the most stress-sensitive component(s) need to be identified. This can be done by subjecting shoot photosynthetic capacity and nodule functioning to differential levels of stress and partitioning the effects of stress to the components of the symbiosis.

Split root growth systems have been used to study the effects of various stresses on plant growth and root development: salinity (Eaton, 1941; Kirkham et al., 1972); moisture tension (Volk, 1947; nutrient

availability (Gile and Carrero, 1917; Hackett, 1972). Hinson (1975) confined nodulation to half the root system of soybeans grown in vermiculite to study the localized effect of mineral N on nodule development. Such a system is not, however, suitable for studying the effect of salinity on the symbiosis since migration of salts in the rooting medium may create zones of variable osmotic potential, so that nodules and roots will not be exposed to a uniform stress.

This paper describes two experiments using a solution culture, split-root growth system suitable for examining the effects of various stresses on components of the legume-Rhizobium symbiosis. The technique was used for two experiments. The first examines two methods of confining nitrogen-fixing root nodules to one-half of soybean root systems and the effect that nodulation has upon root development, water uptake, and nitrogen fixation. A second examines the effect of applying NaCl to either half or whole root systems on leaf, nodule and root osmotic potential (Ψ_π).

Materials and Methods

Growth System

Steel containers (20 liters) were coated inside and out with an elastomeric protective coating USDA has approved for potable water (Vabar 792, United Coatings). The top of each container was removed except for an 8.0 cm strip along one side (Figure III-1). This strip had eight 2.54 cm holes to receive one end of a PVC elbow (21.0 mm O. D., 6.0 cm sides), packed with sterile vermiculite. A planting hole (10 mm

dia.) was drilled on the joint of each elbow. Planting holes were to receive seedling radicles. The ends of elbows were fitted with a rubber washer to stabilize the elbows which spanned two containers. A sight glass was fitted to each container and calibrated volumetrically to the nearest 0.5 liter. Each container had an aquarium airstone connected to a flexible plastic lateral air line to create a constant splash of solution on the ends of the PVC elbows. The solutions were saturated with oxygen as measured with a YSI oxygen meter. Lids for the containers were made from 2.54 cm sheets of polystyrene. The bottom lid was covered with black polyethylene.

Seeds of soybean (Glycine max (L.) Merr. 'Davis') were treated for 5 min. with 70% ethanol, rinsed, planted hilum down in sterile vermiculite and at 28 C for 48 hours. The tips of the seedling radicles were removed to induce branching and seedlings were then inserted into the planting holes. Sterile water was applied to the seedling to ensure capillarity between the cut radicle and vermiculite. The sides of the elbows were covered with foil to maintain the same temperature on both sides.

Preparation of Inoculum

Cultures of Rhizobium japonicum, ineffective strain SM-5 (W. J. Brill, University of Wisconsin, Madison) and an effective strain USDA 110 (USDA, Beltsville, Md.) were grown in yeast extract mannitol broth and counted by the drop plate method (Vincent, 1970) then centrifuged for 10 min. at 12,100 x g and 4 C. The cells were resuspended in

water and inoculated into appropriate containers to the final numbers stated for each experiment.

Nutrient Solution

A nitrogen-free nutrient solution was used consisting of: 0.5 mM P; 0.96 mM K; 1.56 mM S; 0.82 mM Mg; .75 mM Ca. Sources were: K_2HPO_4; $MgSO_4.7H_2O$; $CaSO_4$. Micronutrients were added according to Broughton and Dilworth (1971).

Effective vs Ineffective Rhizobium Experiment

Five days after planting roots emerged from elbows into the containers. At this time, designated containers were inoculated with SM-5 at the rate of 1.5×10^6 viable cells/ml of plant nutrient solution. Forty-eight hours later USDA 110 was inoculated at 4.4×10^4 cells/ml in containers to institute as two treatment combinations: 1) USDA 110 on one side and no inoculation on the other, 2) USDA 110 on one side with ineffective SM-5 on the other. Inoculation with SM-5 preceeded that of USDA 110 so that some of the early processes of infection would be complete and reduce the chance of formation effective nodules on the non-nitrogen fixing (SM-5) side. Containers were arranged in a completely randomized design and replicated three times. Both experiments were carried out in a glasshouse during the months of June and July. Solution pH was monitered but never declined below 5.8. Solution temperatures ranged from 26 C - 28 C at 2 p.m. Nutrient solutions were changed at 20 and 40 days from planting followed by harvest at 60 days.

Containers were inoculated to the original cell density with the appropriate Rhizobium, after each solution change.

At harvest, the containers were drained, shoots were cut and dried at 60 C. Roots were removed and immediately incubated in 2.3 liter plastic vessels containing 5% acetylene. Ethylene production was determined by gas chromatography. Nodules were removed from roots, oven dried, counted and weighted.

Salinization of Half-Root Systems

The planting procedure was identical to that in the first experiment. Solutions were changed at 21 and 34 days from planting. Each container of a pair was inoculated with either SM-5 or USDA 110.

Salt treatments were instituted at Day 34 by adding NaCl to designated containers over a 48-hour period to a final concentration of 120 mM NaCl (normal solution osmotic pressure (OP) = -0.15 bar, salinized solutions OP = -5.5 bar). Four combinations of salt treatments to half-root systems were instituted: 1) No salt to either side, 2) salt to the non-nitrogen fixing half-root system (SM-5), 3) salt to nitrogen-fixing half-root system (USDA 110), 4) salt to both half-root systems.

The osmotic potential ($\Psi\pi$) of the most recently expanded trifoliate, nodules and roots were sampled at Day 45. Nodule and root samples were blotted dry and each placed in 8 cm lengths of 12 mm I. D. flexible plastic tubing. Leaves were placed directly into a tube. Tubes were plugged at both ends with rubber stoppers after expressing most of the air and then frozen on dry ice. Samples were thawed for 20 minutes,

pressed at 1050 kg/cm^2, and the expressed sap was sampled with filter paper discs which were placed in a Wescor Model 5130 vapor pressure osmometer.

Results

Plants with half their roots inoculated with ineffective strain SM-5 had reduced shoot, root, and nodule dry weights. The number, mass and nitrogenase activity of nodules formed by effective strain USDA 110 was reduced when the other half of the root system had SM-5 nodules (Table III-1). Uninoculated half-root systems remained free of nodules. Development of half-root systems was stimulated by inoculation with USDA 110. Water consumption followed a similar pattern. No nitrogenase activity was detected on either the uninoculated or the ineffectively nodulated half-root system.

Leaf $\Psi\pi$ (-10.3 and -11.1 bar) of plants with half of their root system exposed to 120 mM NaCl was not different than the control (-9.3 bar) (Table III-2). Leaf $\Psi\pi$ was reduced to -17.9 bar when both root halves were exposed to salt. Nodule $\Psi\pi$ was a function of the solution to which they were exposed. When USDA 110 nodules were exposed to NaCl nodule $\Psi\pi$ was -10.9 bar and -12.2 bar respectively for half and whole-root salt treatments. Ineffective nodules (SM-5) had nodule $\Psi\pi$ of -9.3 bar for both half and whole-root salt treatments. $\Psi\pi$ of non-salinized USDA 110 nodules was -7.8 bar and -6.1 bar for non-salinized SM-5 nodules. Water absorption by half-root systems was reduced by the addition of 120 mM NaCl. This reduction was compensated for by increased uptake on the non-salinized side.

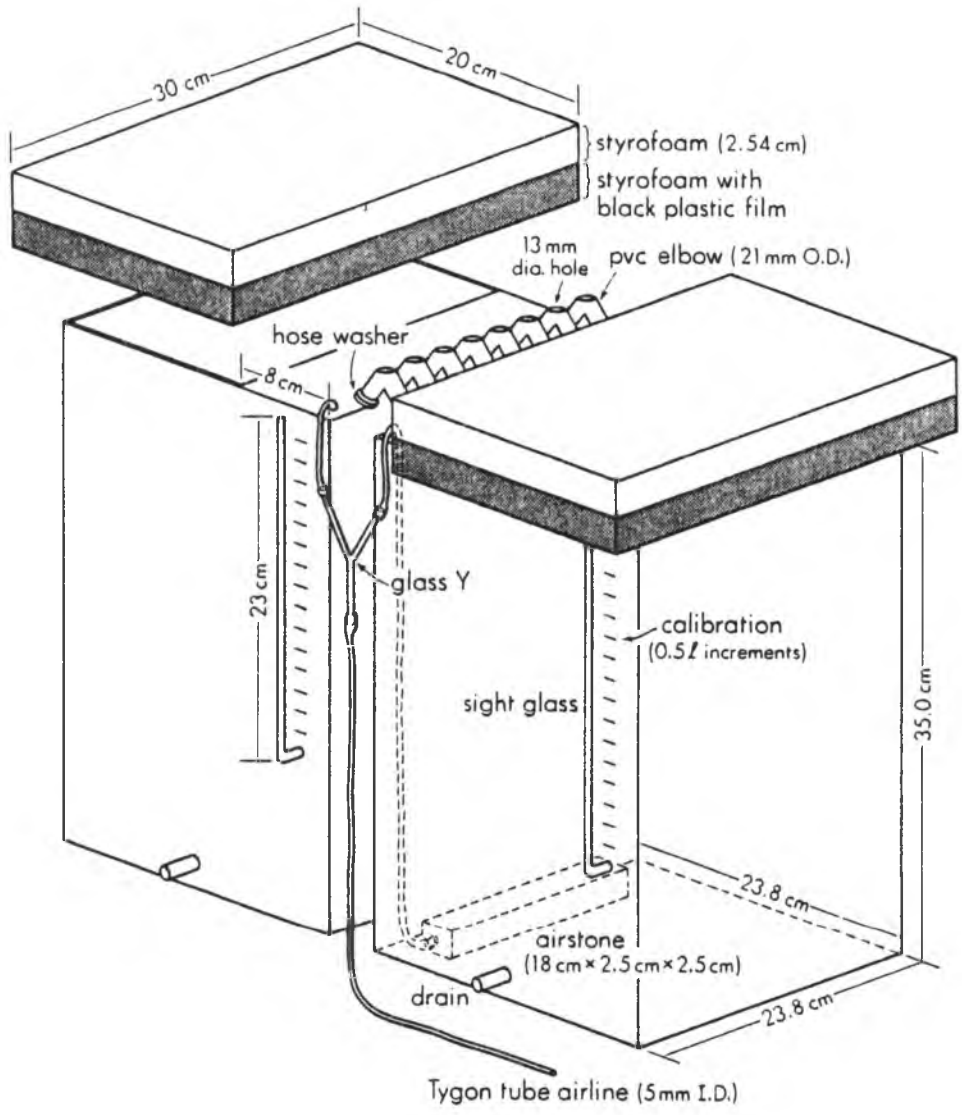

FIGURE III-1. -- A split-root growth system.

TABLE III-1. -- Effect of inoculating soybean half-root systems with effective and ineffective strains of *Rhizobium japonicum* on shoot weight, root and nodule development, nitrogenase activity and total water use.[a]

Inoculation of Half-Root System	Dry Weight			Nodule Number	Nitrogenase Activity	Water Consumption
	Shoot	Root	Nodule			
	g/pot			no/pot	μMC_2H_4/pot/h	l/pot
I						
Uninoculated Side		1.2	trace	1	0	13.0
USDA 110 Side		2.0	1.30	621	56	18.0
Total	18.8	3.2	1.30	622		31.0
II						
SM-5 Side		0.5	0.15	424	0	10.0
USDA 110 Side		0.7	0.23	191	11	11.0
Total	5.1	1.2	0.38	615		21.0
$LSD_{.05}$	13.0	0.6	0.54	233	23	5.0

[a]The ineffective strain, SM-5, was inoculated into the designated side 48 hours before the application of the effective strain, USDA 110, to the opposide side

TABLE III-2. -- Effect of 120 mM NaCl added to half-root systems of nodulated soybean on the osmotic potential ($\Psi\pi$) of leaves, nodules, roots and water consumption subsequent to NaCl addition.

Salt Treatment To Half-Root System Growing:		Osmotic Potential ($\Psi\pi$)						
			Nodule		Root		Water Consumption	
USDA 110 Nodules	SM-5 Nodules	Leaves	USDA 110	SM-5	USDA 110	SM-5	USDA 110	SM-5
mM NaCl		bar					liter/day	
0	0	-9.3	-7.8	-6.1	-5.4	-6.0	0.61	0.44
0	120	-10.3	-7.8	-9.3	-5.5	-7.5	1.10	0.23
120	0	-11.1	-10.9	-6.9	-7.1	-5.8	0.32	0.98
120	120	-17.9	-12.2	-9.3	-8.1	-7.1	0.29	0.33
	$LSD_{.05}$	-3.2	-1.2		-1.3			

Discussion

To test the effects of salinity on the integrated processes of the legume-Rhizobium symbiosis, it is necessary to isolate and independently subject photosynthetic and nodule processes to the stress. A growth system suitable for such purposes must meet two requirements. First, all nitrogen-fixing nodules must be confined to a portion of the root system. Secondly, the salinity stress must be uniformly applied to the nodules with a minimal effect on the photosynthetic apparatus.

Solution culture systems are advantageous for salinity studies since uniform osmotic potentials in the root environment can be maintained. By employing a split-root technique which confines rhizobial inoculation and subsequent nodulation to one-half of the root system, it is possible to subject the functional processes of the nitrogenase system to salinity while maintaining the shoot under less stressful conditions. Kirkham et al., (1969) demonstrated that although leaf water potential of Phaseolus vulgaris with half the root system in salinized medium was reduced, the potential more closely resembled the no salt control than the leaf potential of plants with both root halves in salinized media. Using tritiated water, Sprent (1972) concluded that nodules received water en route from roots to leaves. This would imply that nodules are at a lower water potential than surrounding roots. However, given the large amount of phloem transport taking place from shoot to nodule (Minchin and Pate, 1974), it seems plausible that

the osmotic potential of nodules in the localized stress environment of differentially salinized plants may not adjust to their environment.

This study examines two methods of maintaining nitrogen-fixing nodules on half the root system of soybean. In addition, we evaluate the osmotic adjustment of roots, nodules and leaves of plants with differentially salinized root systems.

Effective vs Ineffective Rhizobium Experiment

Inoculation of half the root system with an ineffective strain (SM-5) 48 hours before the introduction of the effective strain (USDA 110) on the opposite side caused a reduction in the number, weight and activity of the resulting effective nodules (Table III-1). Total nodule number (effective and ineffective) however, remained the same regardless of the treatment. Weight of effective nodules was reduced when SM-5 was inoculated to the other side. Apparently, total nodule number on a soybean root system is controlled by the host and strains of Rhizobium compete for limited nodule sites. Competition for nodule sites exists even when there is spatial separation of the strains on the root system. The competitive advantage of SM-5 when inoculated first indicates that events early in the infection and nodule development processes determine what proportion of the nodules will be formed by each strain. When SM-5 was used as a method to confine effective strain USDA 110 to one-half the root system the proportion of total nodule number (USDA 110 nodules plus SM-5 nodules) that was formed by USDA 110 was less than 30%. Singleton and Stockinger (1982) have shown that despite the existence of a compensating mechanism for ineffective nodulation,

effective nodule mass and nitrogen fixation is seriously reduced when effective nodules were less than 50% of the total. The design of the growth system maintained the uninoculated half-root system sufficiently free of contaminant nodules so that the source of symbiotic nitrogen can be isolated and inoculation with an ineffective strain on one side is not necessary.

Root development was stimulated by effective nodules. Root weight was consistently greater on the side where the effective nodules were present compared to uninoculated and ineffectively nodulated sides. Total water consumption followed the same pattern (Table III-1). Increased flow of carbon to active nodules and the increased localized availability of nitrogen from active nodules may be the reason for increased root development. Drew (1975) showed that application of nitrate and ammonium had a localized stimulatory effect on root development. When the source of symbiotic nitrogen is localized there appears to be a similar stimulation of root development.

Salinization of Half-Root Systems

Application of 120 mM NaCl to half-root systems reduced the osmotic potential ($\Psi\pi$) of roots and nodules exposed to these solutions. Nodules were always at a lower potential than their respective roots. Leaf $\Psi\pi$ was more negative than the $\Psi\pi$ of both nodules and roots when only those half-root systems were exposed to NaCl. The potential gradient necessary for transport of fixation products between nodule and shoot is therefore maintained.

Shoots of plants with half their root system in salinized solutions had leaf $\Psi\pi$ values closer to those of the non-salinized control, than to leaf $\Psi\pi$ of plants with the complete root system in salinized solutions. Increased water uptake by the non-salinized half-root system compensated for salinity stress on the opposite half-root system (Table III-2). These results indicate that the nodule system can be subjected to salinity stress while maintaining a relatively unstressed photosynthetic apparatus.

In conclusion, the growth system design described in this paper provides sufficient control of localized nodulation to confine the source of symbiotic nitrogen fixation to half-root systems of soybean. Osmotic adjustment by roots, nodules and leaves of differentially salinized soybeans with split-root systems allows observation of nodule processes under stress when the shoot is under normal conditions. The use of this type of growth system may prove useful in the study of the effects of other soil stress factors on symbiotic processes.

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CHAPTER IV

THE EFFECT OF SALINITY ON THE FUNCTIONAL COMPONENTS OF THE SOYBEAN-*RHIZOBIUM JAPONICUM* SYMBIOSIS

Abstract

A split-root solution culture system was used to partition the effects of salinity on the functional components of the soybean (*Glycine max* (L.) Merr) - *Rhizobium japonicum* symbiosis. Nodules were confined to half of the root system. After nodules were well developed (40 days from planting), salt, at 120 mM NaCl, was applied to the half-root systems in the following combinations: 1) unstressed, no salt to either half-root system; 2) partially stressed shoot and unstressed nodules, salt to only the non-nodulated half-root system; 3) partially stressed shoot and stressed nodules, salt to only the nodulated half-root system; 4) stressed shoot and nodules, salt to both half-root systems.

Osmotic potentials ($\Psi\pi$) of leaves for the four treatments were: 1) -10.2; 2) -11.2; 3) -12.3; and 4) -18.3 bar. Nodule $\Psi\pi$ for the treatments were: 1) -7.0; 2) -7.2; 3) -11.2; and 4) -12.1 bar. While total and specific nitrogenase activity as well as shoot nitrogen content were suppressed slightly in Treatment 3, the greatest reduction in activity and shoot nitrogen was observed when the plant was stressed by having both half-root systems in salt (Treatment 4). The rate of leaf expansion in Treatment 4 was half the rate observed in other treatments. We conclude that reduced nitrogen fixation by nodulated soybeans growing in saline environments was more a result of the effect

of salt on leaf $\Psi\pi$ and expansion, than the direct action of salt on the functional processes of the nitrogenase system.

Introduction

The legume-*Rhizobium* symbiosis is a highly integrated process involving a complex interaction between the host and microorganism. A constant exchange of metabolites takes place between plant photosynthetic organs and root-nodules. Photosynthate enters the nodule by phloem transport where it is either respired for nodule growth, maintenance and reduction of N_2 or re-exported to the shoot as amino compounds (Pate, 1976). The production and flow of amino compounds, as with other assimilates, is regulated and ultimately partitioned to growth points and reproductive sinks in relation to growth potential (Lewis and Pate, 1973). Therefore, any stress which reduces photosynthesis and plant growth will undoubtedly affect nitrogen fixation.

Soil stress may reduce symbiotic activity to levels below the genetic potential of a host-*Rhizobium* strain combination. The stress could reduce the accumulation of symbiotically derived nitrogen by either affecting shoot photosynthesis and yield potential or by directly affecting processes within the nodule. Due to the integrated nature of the functional processes of the symbiosis it becomes difficult to assess the relative sensitivity of host and microorganism to stress. By identifying which components or processes of the symbiosis is most sensitive to the soil stress, efforts to increase nitrogen fixation in these environments may then focus on the proper component.

Sprent (1972) observed a 50% decline in nitrogenase activity within 19 minutes of exposing the entire nodulated root system of soybean plants to 120 mM NaCl. Exposure of detached soybean nodules to 1 M NaCl also depressed nitrogenase activity.

Lauter et al., (1981) showed that salinity adversely affected nodule weight and total nitrogenase activity of chickpea (Cicer arietinum L.). Similar results were obtained for soybean and alfalfa (Medicago sativa L.) by Bernstein and Ogata (1966) and for Glycine wightii by Wilson (1970). Since salinity is known to reduce photosynthesis and growth of most plants (Gale, 1975), the above studies do not determine how salinity limited the symbiosis.

This study was conducted to assess the effects of salinity on the components of the soybean-Rhizobium japonicum symbiosis. We partitioned the effects of salinity on nitrogen fixation into effects that directly relate to nodule function versus indirect effects which are mediated through the host photosynthetic apparatus.

Materials and Methods

Plant Culture

The split-root solution culture growth system which confined the nodules to half the root system of soybean (Glycine max (L.) Merr C. V. Davis) and the planting procedure have been described earlier (Chapter III). At planting NH_4NO_3 (7.1 mM N) was added to the basal nutrient solution: (mM) 0.5 P; 0.96 K; 1.56 S; 0.82 Mg; 0.75 Ca. Solutions were changed 22 days from planting and one-half of the

split-root system was inoculated with Rhizobium japonicum strain USDA 110 (USDA Beltsville MD) at a density of 9.7×10^5 cells/ml of culture solution. The opposite half of the root system remained uninoculated. NH_4NO_3 (.75 mM N) was added to the basal nutrient solution of the uninoculated half to supply the plant with nitrogen during nodule development. Significant nodule development was observed 40 days from planting. At this time solutions were changed, nodulated sides reinoculated (7.1×10^4 cells/ml) and NaCl was introduced over a two-day period to designated containers to a final concentration of 120 mM NaCl. No nitrogen was supplied to plants after treatment initiation. Solutions were again changed at Day 46, reinoculated (1.7×10^5 cells/ml) and designated containers re-salinized. Plants were harvested at 50 days from planting.

Salinity Treatments to Half-Root Systems

Four treatment combinations between the source of symbiotic nitrogen and salinity were instituted and are displayed in Table IV-1. Treatments were arranged in a completely randomized design and replicated three times.

Measurement of Leaf Expansion

Leaflets of the most recently emerged trifoliate of three plants selected at random were tagged and their initial leaf area determined with a Licor area meter. The initial measurement was performed 3 days

after the initiation of salinity treatments. The same trifoliates were measured 5 days later. Results were expressed as expansion in cm^2/day.

Measurement of Osmotic Potential ($\Psi\pi$)

Eight days after treatment initiation leaf, nodule and root were determined. The most recently expanded trifoliate of three plants not used for measurement of leaf expansion were removed, and nodules and root samples blotted dry and placed in 9 cm lengths of Tygon R-3603 tubing (12 mm I. D., 2 mm wall). The tubes were sealed with rubber stoppers and frozen on dry ice. Tubes were thawed (20 minutes), pressed at 1052 kg/cm^2, and the expressed sap sampled with filter paper discs which were placed in a Wescor 5130 vapor pressure osmometer.

Harvest

Shoots were cut and oven dried at 65 C. Root halves were removed, placed in 2 liter plastic bottles, and incubated for 30 minutes in 5% acetylene. Ethylene production was determined by gas chromatography.

Roots and nodules were separated and oven dried. K and Na contents of nodules were determined by flame photometry from water extracts of ground tissue. Chloride in nodules was determined potentiometrically using the titrating method in conjunction with an Ag-AgCl electrode and a glass reference electrode (Chapman and Pratt, 1971).

Results

Shoot dry weight and the concentration of N in the shoot declined when half- and whole-root systems were exposed to NaCl (Table IV-1).

Leaf osmotic potential ($\Psi\pi$) of plants with only half the root system salinized was between 1 and 2 bar lower than the non-salinized control. With the whole-root system salinized leaf $\Psi\pi$ was 8 bar lower than the control (Table IV-2). Nodules exposed to salt had a $\Psi\pi$ 4 bar lower than non-salinized nodules.

Nitrogenase activity was reduced when the nodulated half-root system was exposed to salt. However, there was a much greater decline in nitrogenase activity when the whole-root system was salinized (Table IV-3). Specific nitrogenase activity was reduced when nodules were exposed to salt. Specific activity of nodules on plants with both half-root systems salinized was approximately half of that of nodules on plants with only the nodulated half salinized (Table IV-2).

Leaf expansion, while affected slightly by salinizing either half the root system, was reduced by 50% when both half-root systems were salinized (Table IV-2).

Data for the concentrations of Na^+, Cl^- and K^+ in shoots and nodules are given in Table IV-2.

TABLE IV-1. -- The effect of NaCl (120 mM) applied to half-root systems of soybean on shoot dry weight and shoot nitrogen.

	Treatment to Half-Root System[a]		Shoot Dry Weight	Shoot Nitrogen	
	Nod	Non-Nod			
			g pot^{-1}	g pot^{-1}	%
1.	-	-	150.6	4.1	2.69
2.	-	+	118.3	3.2	2.70
3.	+	-	130.9	2.6	2.01
4.	+	+	80.5	1.8	2.29
	$LSD_{.05}$		34.9	1.2	0.53

[a]Half-root systems nodulated (Nod) or non-nodulated (Non-Nod) in either normal nutrient solution (-) or nutrient solution plus 120 mM NaCl (+). Salt treatments instituted at Day 40 from planting until harvest at 50 days.

TABLE IV-2. -- The effect of shoot and nodule osmotic potential ($\Psi\pi$) on nitrogenase and leaf expansion of soybean.

	Treatment to Half-Root System[a]				Nitrogenase Activity		Expansion of new
	Nod	Non-Nod	$\Psi\pi$ Leaf	$\Psi\pi$ Nodule	Total[b]	Specific[c]	Trifoliate
			bar		μMC_2H_4		$cm^2\ day^{-1}$
1.	-	-	10.2	7.0	319	67	34
2.	-	+	11.2	7.2	217	57	31
3.	+	-	12.3	11.2	168	43	31
4.	+	+	18.3	12.1	47	23	16
	$LSD_{.05}$		3.9	1.5	149	27	12

[a]Half-root systems nodulated (Nod) or non-nodulated (Non-Nod) in either normal nutrient solution (-) or nutrient solution plus 120 mM NaCl (+). Salt treatments instituted at Day 40 from planting until harvest at Day 50.

[b]Total nitrogenase activity of nodulated half-root systems: $\mu MC_2H_4 pot^{-1} h^{-1}$.

[c]Specific nitrogenase activity: μMC_2H_4 (g nodule dry weight)$^{-1} h^{-1}$.

Discussion

Studies on the effect of salinity on the legume-Rhizobium symbiosis have always subjected the entire symbiotic system to the stress. Increasing salinity results in a reduction of nodule number, nodule weight and total N fixed by Glycine wightii (Wilson, 1970). Bernstein and Ogata (1966) and Lauter et al., (1981) observed similar effects of salinity on nodulation and nitrogen fixation by Glycine max and Cicer arietinum respectively. In both studies NO_3-fed plants subjected to salinity had higher relative yields than similarly treated symbiotic plants. This suggests that the processes of the symbiosis are relatively more sensitive to salinity than the mechanisms of uptake and metabolism of mineral N. The sensitivity of particular symbiotic processes cannot be determined from these studies since both the shoot and nodule were stressed simultaneously. To identify the symbiotic process most susceptible to salinity, it is necessary to subject shoot and nodules to salinity stress independently.

Huang et al., (1975b) partitioned the effects of soil moisture tension to photosynthesis and nodule function of soybean. Photosynthesis of drought stressed plants was enhanced by CO_2 enrichment which resulted in an increase in nitrogenase activity. Their methodology, however, requires a great deal of preliminary investigation and more complex procedures than the split-root technique used in this study.

Since symbiotic nitrogen fixation is dependent on recently-fixed photosynthate (Minchin and Pate, 1974; Huang et al., 1975a) root

TABLE IV-3. -- The effect of NaCl (120 mM) applied to half-root systems of soybean on the concentration of shoot and nodule Na, Cl, and K.

	Treatment to Half-Root System[a]		Na^+		Cl^-		K+	
	Nod	Non-Nod	Shoot	Nodule	Shoot	Nodule	Shoot	Nodule
			%					
1.	-	-	0.05	0.2	0.21	0.03	2.3	2.13
2.	-	+	0.18	0.4	0.88	0.05	2.2	1.72
3.	+	-	0.23	3.7	1.05	0.44	1.9	0.88
4.	+	+	0.98	3.7	3.35	0.49	2.5	0.82
	$LSD_{.05}$		0.36	0.15	0.59	0.16	0.22	0.43

[a]Half-root systems nodulated (Nod) or non-nodulated (Non-Nod) in either normal nutrient solution (-) or nutrient solution plus 120 mM NaCl (+). Salt treatments instituted from Day 40 from planting until harvest at Day 50.

environment stress which reduces photosynthesis (or the translocation of photosynthate) to the nodules will have a rapid effect on nodule function. Despite osmotic adjustment of roots and shoots to reduced water potentials in the root environment (Bernstein, 1961), photosynthetic assimilation of carbon by glycophytes such as soybean is adversely affected (Sung and Krieg, 1979; Jensen, 1975; Gale, 1975; Turner et al., 1978). Sung and Krieg (1979) concluded that photosynthesis was reduced with water stress in sorghum and cotton prior to any reduction in translocation rates. Reduced photosynthesis by plants in saline environments is characterized by reduced leaf expansion (Jensen, 1975; Hawker and Walker, 1978) and increased leaf resistance to CO_2 assimilation (Jensen, 1975).

The osmotic potential of shoots and nodules (Table IV-2) indicate that leaves and nodules of the same plant could be subjected to differential salinity stress. Nodules of Treatments 2 and 3 are functionning at a 4 bar $\Psi\pi$ differential while leaves of both treatments have approximately the same $\Psi\pi$. Treatments 3 and 4 have nodules at the same $\Psi\pi$ but leaves carrying on growth and photosynthesis at a 5 bar difference. Turner et al., (1978) and Huang et al., (1975a) found that photosynthetic rates in soybean were reduced substantially when leaf water potentials were below 14 bar.

Exposing either the nodulated or non-nodulated half of the root system to salt resulted in a reduction in both shoot dry weight and total shoot N (Table IV-1). When only the nodulated half was salt stressed (Treatment 3) shoot N concentration was reduced. Apparently,

the stressed nodules ($\Psi\pi$ = -11.2 bar) could not supply adequate nitrogen to meet the growth requirements of the relatively unstressed shoot. The greater percent N in the shoot when both half-root systems were stressed (Treatment 4) indicates that it was not N availability that was limiting shoot growth in this treatment.

The direct effects of salinity on nodule function can be determined by comparing nitrogenase activities of Treatments 2 and 3. Total and specific nitrogenase activity was reduced when salinization of nodulated half-root systems resulted in a -4 bar change in nodule $\Psi\pi$. When the whole symbiotic system was salt stressed (Treatment 4), however, a large reduction in nitrogenase activity was observed. This reduction can best be explained by the large reduction in leaf $\Psi\pi$ and leaf expansion rather than nodule $\Psi\pi$.

Low water potentials in the root environment affect both photosynthetic rates (Boyer, 1970) and leaf expansion (Hawker and Walker, 1978). Since shoots are both the source of energy for the nitrogenase system and the "sink" for nodule products, leaf expansion can be considered as a measure of change in photosynthetic capacity and strength of the "sink" for fixed nitrogen. The effect of reducing leaf $\Psi\pi$ on leaf expansion is illustrated in Table IV-2. Although leaf expansion rates were slightly affected when one-half the root system was exposed to salt and a leaf $\Psi\pi$ declined by 1 or 2 bar, a 50% reduction in expansion rate was observed when leaf $\Psi\pi$ declined to -18.3 bar. Nitrogenase activity in the soybean-*R. japonicum* symbiosis is therefore limited by salinity through a reduction in leaf $\Psi\pi$ and shoot

growth. The nitrogenase system can withstand substantial reductions in $\Psi\pi$ from exposure to extremely saline environments provided the shoot remains relatively unstressed.

Tissue analysis of shoots and nodules help to explain these results (Table IV-3). Nodules, like roots (data not shown), accumulated more Na^+ and excluded Cl^- relative to the shoot. Uptake of Na^+ by nodules was negatively correlated with nodule K^+. Shoot K^+ was not greatly affected by the salinity treatments. Our results show that nodule activity can proceed at near normal rates despite the presence of high concentrations of Na^+, and an apparent reduction in K^+.

In conclusion, differentially salinized soybeans with nodules confined to one-half the root system provide a simple system in which the processes of the symbiosis may be subjected to stress independently. Plants with half their roots exposed to salt had leaf $\Psi\pi$ and shoot yield potentials approximately equivalent to non-salinized controls. Nodule $\Psi\pi$ was a function of the $\Psi\pi$ of the solutions to which they were exposed regardless of leaf $\Psi\pi$. Nitrogenase activity of salinized nodules was more a function of the degree of stress in the shoot rather than the direct effect of salt on nodule processes. Even with the high concentration of salt used in this experiment (120 mM NaCl), there appears to be little direct effect of salt on nodule function. Reduced nitrogenase activities observed in plants with the whole root system salt stressed was due to salt limiting leaf expansion, shoot growth and demand for nitrogen. The relative tolerance of established nodules to low water potentials may have evolutionary and ecological relevance.

Annual legumes such as soybean may mature using water deep in the soil profile while, as the surface soil dries, nodules must adjust and function in an environment of declining soil matric and osmotic potentials.

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CHAPTER V

THE EFFECT OF SALINITY ON NODULE FORMATION

Abstract

A split-root growth system was employed to evaluate the effect of NaCl on nodule formation by soybean. By applying the salinity stress and rhizobial inoculum to only one-half the root system the effects of salinity on shoot growth were eliminated in the nodulation process. Rhizobium colonization of root surfaces was not affected by the salt treatments (0.0, 26.6, 53.2, and 79.9 mM NaCl). While shoot dry weight remained unaffected by the treatments, total shoot N declined from 1.26 g N/pot at 0.0 mM NaCl to 0.44 g N/pot at 79.9 mM NaCl. The concentration of N in the shoot declined from 3.75% N (control) to 1.26% N at 79.9 mM NaCl. Reduced shoot N was attributed to a sharp decline in nodule number and dry weight. Nodule number and weight were reduced by approximately 50% at 26.6 mM NaCl, and by more than 90% at 53.2 and 79.9 mM NaCl. Nodule development, as evidenced by the average weight of a nodule was not as greatly affected by salt as was nodule number. Total nitrogenase activity (C_2H_2 reduction) declined in relation to nodule number and dry weight. Specific nitrogenase activity, however, was less affected by salinity and was not depressed significantly until 79.9 mM NaCl. Isolates of R. japonicum reisolated from nodules formed at 79.9 mM NaCl did not show increased nodulation of roots under salt stress than did nodule isolates from normal media. The early steps in nodule initiation are, therefore, extremely sensitive

to even low concentrations of NaCl. The sensitivity is not related to rhizobial survival and is probably due to the salt sensitivity of root infection sites.

Introduction

The successful initiation of nodulation and nitrogen fixation by a genetically compatible legume-*Rhizobium* combination has two prerequisites: colonization of root surfaces and attachment of rhizobia to roots followed by infection of root hairs. Stress factors such as soil salinity may have an adverse effect on these two processes and limit nitrogen fixation by reducing nodule number.

Rhizobium growth and survival are generally more tolerant in vitro to high osmotic pressures than their respective host legumes (Carr and Ballard, 1979; Lauter et al., 1981). Tu (1981), however, observed reduced colonization of soybean root surfaces by *Rhizobium japonicum* when plants were grown in a salinized culture medium.

Legumes grown in saline environments have reduced yield potential and reduced numbers and weight of root nodules (Tu, 1981; Lauter et al., 1981; Balasubramanian and Sinha, 1976; Lakshmi et al., 1974; Wilson, 1970). There were, however, serious limitations in the above studies for evaluating the effects of salinity on the early stages of nodule formation. With the exception of the work of Lakshmi et al., (1974), inoculation of seedlings with *Rhizobium* preceded the salinization of the rooting medium. It is likely that in these studies some critical steps of rhizobial attachment and infection thread formation could have

occurred prior to the introduction of the salt stress. Plant growth in the study of Lakshmi et al., (1974) was so restricted that even non-salinized control plants of Medicago sativa had less than two nodules per plant.

All the previous work concerning the effects of salinity on nodule initiation suffer from the fact that plant yield potential was affected by the salinity treatments. Reduced shoot growth resulting from non-soil related stress such as low light intensity also reduces nodule number (Sprent, 1973). This relationship between shoot yield potential and nodule initiation requires therefore, that the stress imposed upon the site of nodule initiation does not affect shoot growth.

In this paper, the sensitivity of rhizobial colonization of root surfaces and nodule initiation to salinity were examined. Plant growth potential as a confounding variable was eliminated by employing a split-root growth system described in Chapter III. This system permitted the application of increasing salt concentrations to the site of root-Rhizobium interaction without affecting plant growth potential. In addition, isolates which formed nodules under high salt conditions were tested to determine whether these were variants (mutants) capable of producing nodules under salinity stress.

Materials and Methods

Plant Culture

Eight soybean (Glycine max (L.) Merr. 'Davis') were planted in a split-root solution culture growth system (20 liter capacity on each side) as described in Chapter III. The nutrient solution consisted of: 0.5 mM P; 0.96 mM K; 1.56 mM S; 0.82 mM Mg; .75 mM Ca. Sources were: K_2HPO_4; $MgSO_4$. $7H_2O$; $CaSO_4$. Micronutrients were added according to Broughton and Dilworth (1971). Nitrogen (3.57 mM) NH_4NO_3 was added to each container at planting. Eighteen days after planting solutions were replaced with N-free nutrient solution. Salt was added to one container of the split-root assembly at concentrations of: 0.0, 26.6, 53.2, or 79.9 mM NaCl. Two hours later the salinized side was inoculated with Rhizobium japonicum strain USDA 110 at a viable cell density of 1.2×10^6 cells/ml nutrient solution. Two days later 0.7 mM N as NH_4NO_3 was added to the uninoculated side to maintain leaf area and vigorous plant growth during the early stages of nodule formation. Solutions were sampled for enumeration of Rhizobium and changed at 46 days from planting. Samples were frozen. Re-inoculation of salinized half-root systems was at 9.9×10^4 cells/ml solution. The four treatments were replicated three times in a completely randomized design. Water uptake was monitered daily and solutions were replenished when the level fell by 2 liters.

Harvest

Shoots and roots were cut. Roots were immediately placed in 5.0% acetylene in 2.0 liter plastic containers and incubated for 30 minutes. Ethylene production was determined by gas chromatography. Nodules and roots were subsampled for reisolation and a Rhizobium colonization study and stored at 4 C. The remaining nodules were removed from roots and nodules, roots, and shoots were dried at 65 C. Shoot N was determined by micro-Kjeldahl.

Rhizobium Culture

Yeast extract mannitol (Vincent, 1970) broth cultures were counted by the drop plate method (Vincent, 1970) and then centrifuged at 12,100 x g and 4 C. Cells were resuspended in water for inoculation.

Enumeration of Rhizobium in Plant Nutrient Solution

Frozen nutrient solution samples were thawed, 1 ml aliquots were diluted in distilled H_2O and filtered through a 0.4 m Nucleopore polycarbonate membrane filter that had been stained with Irgalan Black. Filters were treated with USDA 110 fluorescent antibody (F. A.) prepared according to the methods of Schmidt et al., (1968). Cell counts were made by fluorescence microscopy.

Colonization of Root Surfaces by Rhizobium

Root sections (2 cm) that had been subsampled and stored at 4 C were incubated for 30 minutes in an Eriochrome Black solution prepared

according to the method of Goldman (1968) with the exception that dimethyl-sulfoxide (DMSO) was substituted for N, N-dimethylformamide. Roots were rinsed in water until all excess dye was removed, then treated with gelatin-rhodamine isothiocyanate (Bohlool and Schmidt, 1968). Root sections were then incubated with USDA 110 fluorescent antibody. Random microscope fields (100) were examined for positive antibody reaction. A field with any fluorescent cells was counted as being colonized.

Isolation and Testing of Rhizobium japonicum Strain USDA 110 from Nodules Formed in Highly Salinized Rooting Medium

Isolates from surface sterilized nodules were made from the unsalinized controls and from the few nodules formed when the nodule initiation process was exposed to 79.9 mM NaCl. Isolates were identified as being strain USDA 110 by immunofluorescence microscopy (Schmidt et al., 1968). Two isolates from unsalinized controls and two from the 79.9 mM NaCl treatment were then inoculated to half-root systems with either no NaCl or 79.9 mM NaCl in the rooting medium. The plant growth system has been described earlier. Inoculation was at 20 days from planting. Nutrient solutions were changed prior to inoculation and NaCl at 79.9 mM was added to designated half-root systems 2 hours prior to inoculation. Cell densities were 9×10^5/ml nutrient solution for both strains. Nitrogen (0.35 mM) as NH_4NO_3 was added to the uninoculated side at 23 and 27 days from planting.

Results

There was no treatment effect on the fluorescent antibody counts of Rhizobium japonicum strain USDA 110 in the nitrogen-free nutrient solution (Table V-1). Cell densities in all treatments declined from 10^6 cells/ml at inoculation to 10^4 cells/ml at 46 days from planting. Colonization of roots was similarly not affected by salinization of the rooting medium. Roots exposed to the most concentrated level of salt were heavily colonized.

Total shoot N and concentration of N in the shoot declined as the concentration of NaCl applied to the half-root system increased (Figure V-1). Shoot weight was not affected by the salinity treatments.

Exposing the infection process to NaCl caused a sharp reduction in nodule number, nodule mass, and total nitrogenase activity (Figure V-2). The decline was considerable even at the lowest concentration of salt (26.2 mM NaCl). Specific nitrogenase activity was more resistant to salt stress; a significant reduction was not evident except at the highest level of salt employed (79.9 mM NaCl).

Nodule development expressed as the average weight of a nodule was reduced as much as 50% by exposure to NaCl (Figure V-2 inset). The relative effect of salinity on nodule development was not as great as the effect of salinity on nodule number.

Exposure of a half-root system to increasing concentrations of NaCl reduced root growth by that side (Figure V-3). Reduced growth on the salinized side was completely compensated for by increased

root proliferation on the non-salinized side. Water uptake by the two sides followed a similar trend.

Isolates made from root-nodules that developed in the 0.0 and 79.9 mM NaCl treatments in the first experiment were not different in their ability to nodulate the host with 79.9 mM NaCl in the rooting medium (Table V-2). With no NaCl in the medium, the isolates from 79.9 mM NaCl treatments show increased total and specific nitrogenase activity.

Discussion

Nodule initiation in the legume-Rhizobium symbiosis involves a complex interaction between host root, rhizobial strain and the environment. The processes of attachment and proliferation of rhizobia on root surfaces followed by infection thread formation in susceptible host root cells may be sensitive to the environment. Since the host supply of photosynthate required for shoot growth, nodule initiation, development and nodule function is also sensitive to salinity (Jensen, 1975), evaluation of symbiotic processes exposed to stress requires that the processes be independently subjected to the stress. By utilizing a split-root growth system we were able to independently subject the nodulation process of the soybean-Rhizobium japonicum symbiosis to salinity and eliminate shoot stress as a variable in nodule initiation. Shoots of plants with their roots split between normal and salinized mediums have approximately the same shoot growth as plants with all their roots in a normal rooting medium (Kirkham et al., 1969).

The reduction in nodule initiation caused by NaCl salinity was not related to the survival of strain USDA 110 within the range of NaCl concentrations used in this experiment (Table V-1). This agrees with results reported by Carr and Ballard (1979) who reported that many Rhizobium could survive salt solutions approaching the concentration of sea water. Tu (1981) claimed that rhizobial colonization of soybean roots was a limiting factor in nodule formation; however, the salt concentration employed (179 mM NaCl) was excessive for the growth of soybean (Chapter IV). This study shows that nodule initiation is adversely affected by NaCl concentrations which are not inhibitory to rhizobial colonization.

The accumulation of N in the shoot was greatly reduced by imposing salinity treatments to half-root systems prior to the introduction of the rhizobial inoculum (Figure V-1). Total shoot N was correlated with shoot N concentration rather than shoot dry weight.

Nitrogen stress in the shoot was due to insufficiency in numbers and mass of nodules caused by increasing salinity in the rooting medium during nodule initiation. Our experiment shows that the process of nodule initiation in soybean is extremely sensitive to NaCl. A reduction in nodulation of 50% compared to maximum nodule number and mass occurred with only 26.6 mM NaCl. Tu (1981) found that up to 102 mM NaCl in the rooting medium of soybean did not result in a decline in nodule number. Inoculation of the rooting medium with Rhizobium japonicum, however, was performed prior to the institution of salinity treatments. Sensitive steps in the nodule initiation process may have already been

completed by the time roots were exposed to salt. Hence, Tu's experiment examined the effects of NaCl on nodule development.

The development of nodule tissue following infection is more resistant to salinity (Figure V-2 inset). Nodule size at 79.9 mM NaCl was 50% of the no-salt control while nodule number was less than 10% of the control. It has been shown that reduced numbers of nodules on soybean roots is compensated for by an increase in the average weight of a nodule so that total nodule weight remains approximately constant as the number of nodules declines (Singleton and Stockinger, 1982).

Apparently, NaCl stress limits this compensatory response so nodule weight declines with nodule number.

Total nitrogenase activity was a function of nodule number and mass (Figure V-2). The nitrogenase system was more tolerant of exposure to NaCl. Nodule specific activity was not affected except at the highest level of salinity (Figure V-2). This is consistent with the results of Chapter IV which showed that nodule function was relatively resistant to salt stress.

Data for root growth and water uptake in the split-root system explain how plants with one-half the root system exposed to salt can have shoot growth similar to the non-salinized control (Figure V-3). Reduced root growth and water uptake by salinized half-root systems were compensated by increased root growth and water uptake by the non-salinized side. Shoot osmotic potential of split-root plants is relatively unaffected when even 120 mM NaCl is applied to only one-half the root system of soybean (Chapter III).

Some workers have tested a number of rhizobial strains to determine if strain selection could increase nitrogen fixation in saline environments (Lauter et al., 1981; Bhardway, 1975). Isolates were made from nodules formed in the 79.9 mM NaCl treatment of the first experiment to determine whether these isolates were variants of the original culture and capable of increased nodule formation under salinity stress. Although the isolates from the salinity treatment formed more nodules in 79.9 mM NaCl (Table V-2) than isolates made from the non-salinized treatment, the difference was small and nitrogenase activity was not appreciably enhanced. Isolates from the non-salinized control produced more nodules and greater nodule weight in the 0.0 mM NaCl treatment of this experiment yet had substantially reduced acetylene reduction activity than isolates from 79.9 mM NaCl. The isolates made from the first experiment have variable symbiotic properties; this did not include, however, an increased ability to form nodules in saline culture medium.

In conclusion, the processes involved in nodule formation are extremely sensitive to NaCl. Even low concentrations (26.6 mM NaCl) cause significant reductions in nodule number and weight. As a result, shoot nitrogen yield was limited by insufficient nodule tissue to meet the N requirements of unstressed shoots. Nodule development as evidenced by the average weight of a nodule and nodule function (specific nitrogenase activity) were relatively less sensitive to salt than nodule initiation. The ability of Rhizobium japonicum strain USDA 110 to survive and colonize root surfaces was not affected by salinity. The use of isolates made from the high salt treatment as inoculum in a

saline environment indicates that nodulation failure was due primarily to the effects of salinity on plant root infection sites.

TABLE V-1. -- The effect of NaCl in the rooting medium on survival of Rhizobium japonicum strain USDA 110 and colonization of soybean roots.

NaCl (mM)	Log no. cells (ml)	Fields colonized (percent)
0.0	4.11	92
26.6	3.96	76
53.2	4.36	82
79.9	4.02	91
LSD .05	N. S.	

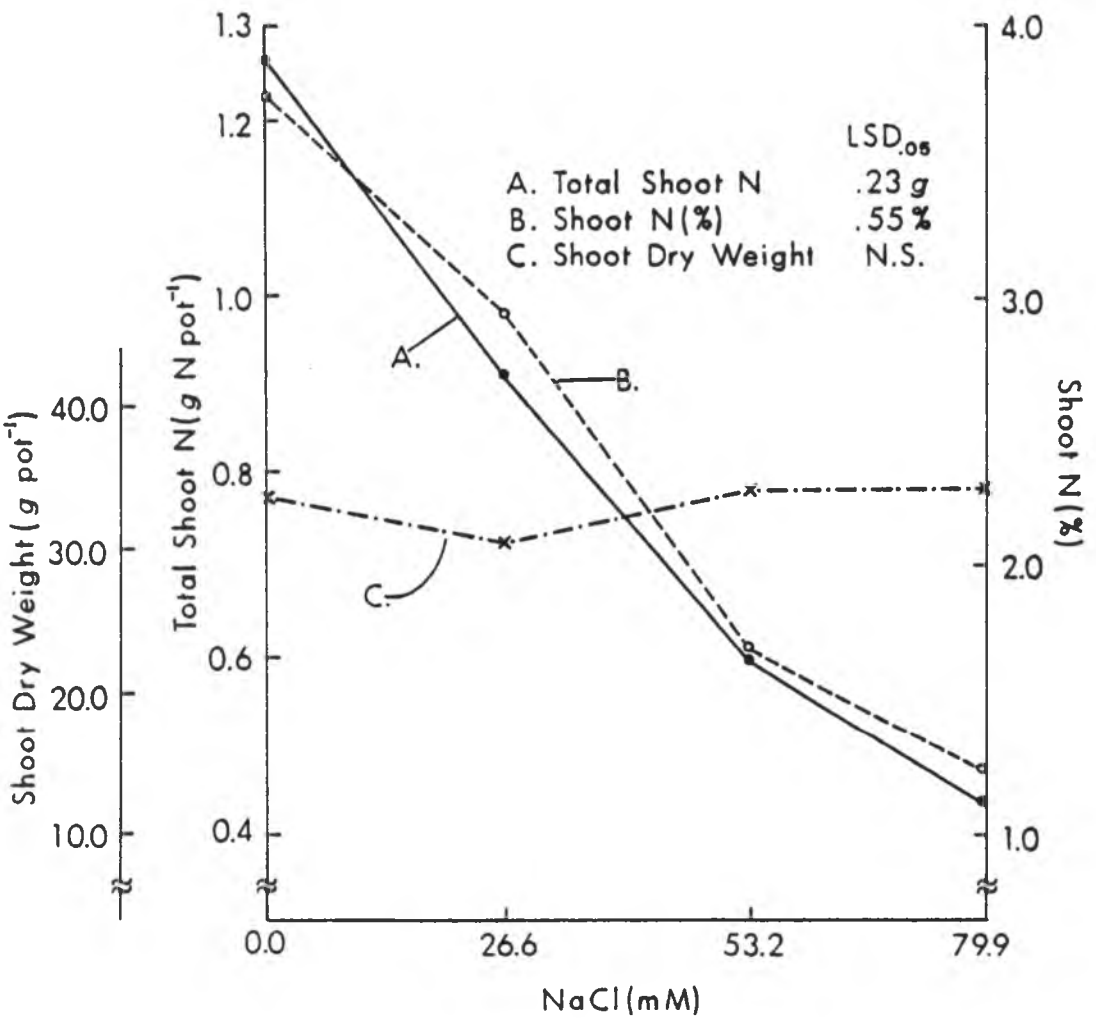

FIGURE V-1. -- Effect of NaCl at inoculation on shoot dry weight shoot N and the concentration of N in the shoot.

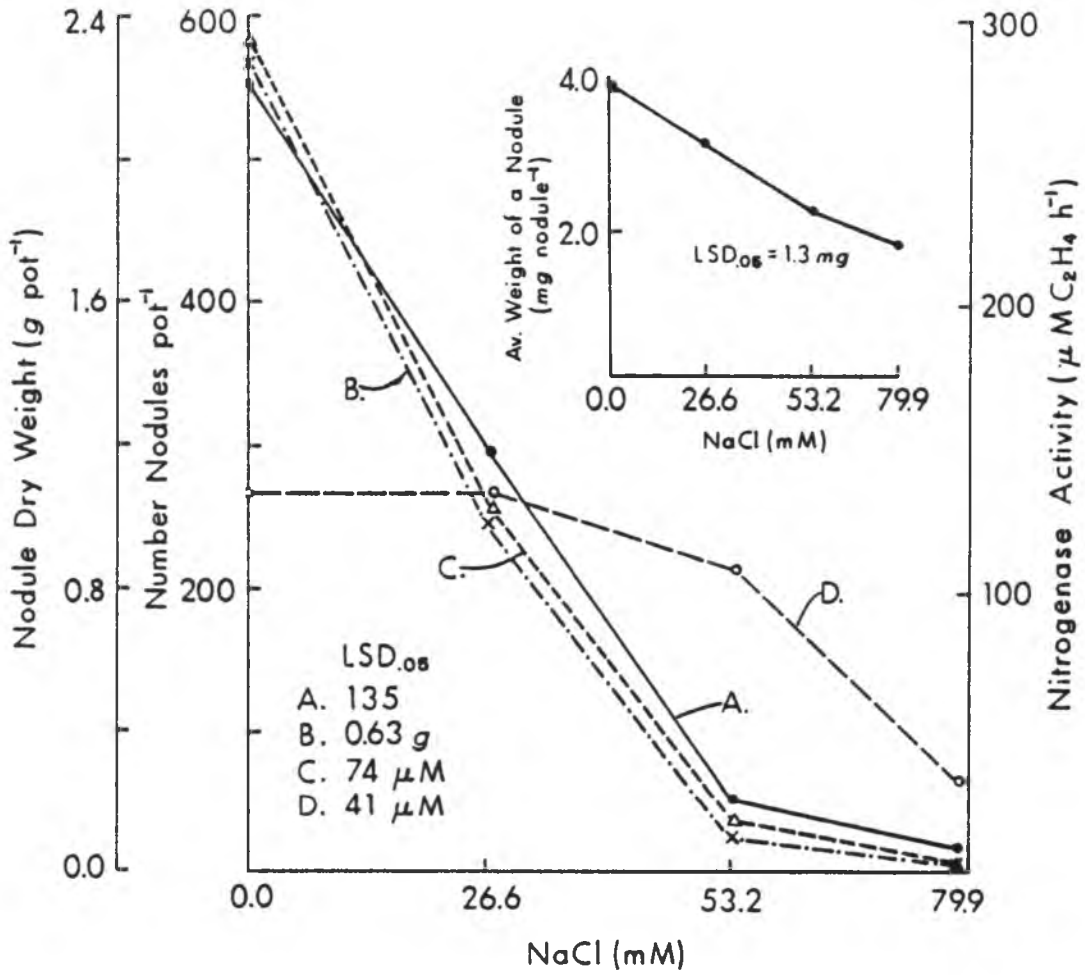

FIGURE V-2. -- Effect of NaCl at inoculation on Nodule Number (A), Nodule Dry Weight (B), Total Nitrogenase Activity (C), Specific Nitrogenase Activity (D) and Nodule Development.

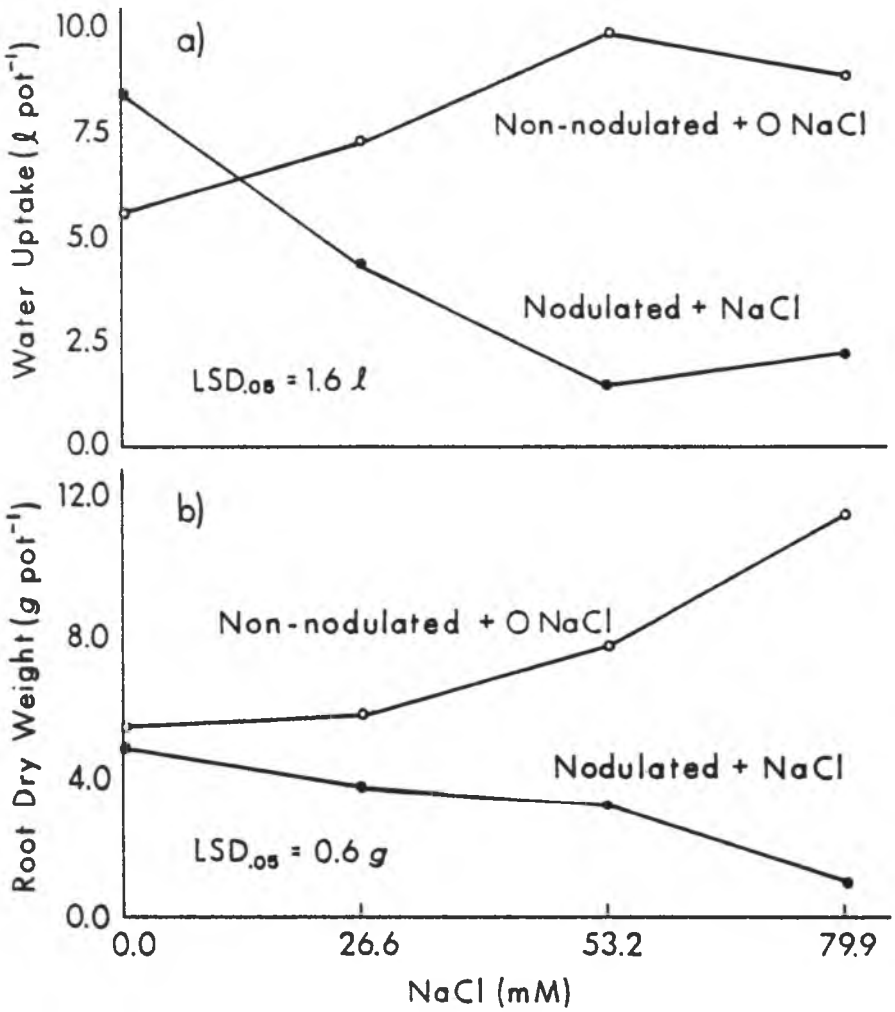

FIGURE V-3. -- Effect of NaCl to half-root systems on root weight and water uptake.

TABLE V-2. -- Relative salt tolerance of isolates from high salt treatments to form nodules in saline solutions.

Isolate from:	Half-root exposed to:	Nodule number	Nodule dry weight	Nitrogenase activity 1	Nitrogenase activity 2
——mM NaCl——		number pot^{-1}	g pot^{-1}		
0.0	0.0	1229	2.97	176	65
79.9	0.0	974	2.55	271	108
0.0	79.9	47	0.25	6	57
79.9	79.9	10	0.18	2	59
	$LSD_{.05}$	411	0.76	34	

[1]Total nitrogenase activity, Mol C_2H_4 pot^{-1} hr^{-1}.

[2]Specific nitrogenase activity, Mol C_2H_4 (g nodule)$^{-1}$ hr^{-1}.

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CHAPTER VI

GENERAL DISCUSSION

The results of these experiments have practical implications for both management of legume production and research. This chapter discusses some of the more important results in relation to production management and future research.

Although additional research is necessary to determine the length of time that the nodule formation process is susceptible to soluble salts, Chapter V indicates clearly that water management during the early stages of soybean establishment is critical. Efforts to decrease osmotic pressures surrounding the emerging radicle when the early processes of root hair infection occur may result in the formation of adequate numbers of nodules and nodule mass to meet the N requirements of the plant. Although nodule development was affected by NaCl (as evidenced by a decline in the average weight of a nodule with increasing salinity), this effect was relatively unimportant when compared to the effect of NaCl on nodule number. Nodule function in the presence of salinity was also relatively more tolerant of exposure to NaCl than nodule formation. These results imply that once the symbiotic system is established, it is relatively more tolerant of NaCl than is plant development. This emphasizes the need to utilize the highest quality of irrigation waters during establishment of the symbiotic system. Subsequent water management should be designed in relation to crop sensitivity.

The split-root technique described in Chapter III will be useful for the examination of the effects of other soil stress factors on the components of the legume-Rhizobium symbiosis. By independently subjecting the components of the symbiosis to the stress, a determination of relative tolerance of each aspect may be made. Research efforts and funding may then be directed toward the improvement of the tolerance of the most sensitive component.

Another use of the split-root technique is in the study of competition between strains of Rhizobium for nodule sites. The data presented in Chapter III suggests that microbe-microbe competition may be separated from plant mediated effects on competition by utilizing the split-root technique.

Results of Chapter II indicate that it is not rhizobial survival in saline environments that limit nitrogen fixation. The tolerance of Rhizobium to salt concentrations approaching 50% sea water suggests that saline solutions may have some use in the long-term storage of Rhizobium.